REMARKS

Status of the Claims

Claims 1, 3-7, 12, 14, 15, 17, 18, 20, 21, 23 and 24 are currently pending in the application. Claims 1, 3-12, 14, 15, 17, 18, 20, 21, 23 and 24 stand rejected. Claim 1 has been amended as set forth herein. Claims 8-11 have been cancelled herein. All amendments and cancellations are made without prejudice or disclaimer. No new matter has been added by way of the present amendments. Specifically, the amendment to claim 1 is supported by original claim 10, which is now cancelled. Reconsideration is respectfully requested.

Rejections Under 35 U.S.C. §§ 102(b) and/or 103(a)

Claims 1, 3-12, 14, 15, 17, 18, 20, 21, 23 and 24 stand rejected under 35 U.S.C. § 102(b) as being anticipated by or, in the alternative, under 35 U.S.C. § 103(a) as being obvious over Hansen, WO 98/54961 (hereinafter referred to as "Hansen"). (*See*, Office Action of May 31, 2006, at page 2, hereinafter, "Office Action"). Claims 8-11 have been cancelled herein without prejudice or disclaimer, thus obviating the rejections as to claims 8-11. Applicants traverse the rejection as to the remaining claims as set forth herein.

In response to Applicants' comments made in the Reply of March 17, 2006, the Examiner directs the Applicants' attention to the fact that the example disclosed in Hansen uses at least two kinds of maize tissue, immature embryos and type I callus. (*Id.* at page 3). Referring to page 17 of Hansen, the Examiner states that there is disclosed the use of immature embryo tissue in addition to type I callus. (*Id.*). The Examiner further comments that although the method at the top of page 19 of Hansen may not explicitly state that centrifugation of the maize tissue is required, the Examiner maintains that one of ordinary skill in the art would know that the embryos would have to be centrifuged to replace the medium. (*Id.*). Furthermore, the Examiner states that the tissues are heated at 45 °C in a water bath, similar to the "heating and/or centrifugation" step recited in claim 1. (*Id.*).

In the response to the previous Office Action, we argued in our Reply of March 17, 2006, that the type I callus described in Hansen is large, hard and dense. We further argued that due to these properties of type I callus, one of ordinary skill in the art would not carry out centrifugation in handling this type of callus.

In response to our argument, the Examiner points out that Hansen uses not only type I callus, but also immature embryos for the transformation.

However, even in handling the immature embryos, those skilled in the art do not carry out centrifugation. The size of immature embryos is about 1 to 2 mm. (*See*, Hansen, at page 17, Example 6). Although immature embryos are not as hard as type I callus, the immature embryos are sufficiently large and heavy, that they sink immediately after being placed in an aqueous medium. Therefore, even when replacing the liquid medium, there is no need to carry out centrifugation. In fact, those skilled in the art do not centrifuge immature embryos. Therefore, until proven otherwise, Hansen cannot be cited for the proposition that its disclosure shows centrifugation of either the type I callus or the immature embryos. As is common knowledge to one of ordinary skill in the art, the immature embryos and the type I callus are not centrifuged.

As further proof of this concept, the Examiner is referred to Frame et al., *Methods in Molecular Biology*, vol. 343, Agrobacterium Protocols, 2nd Ed., volume 1, Chapter 15, Item 3.4, page 192 and Zhao et al., *Transformation of Maize Via Agrobacterium tumefaciens Using a Binary Co-Integrate Vector system: Methods in Molecular Biology*, vol. 318, Plant Cell Culture Protocols, Second Edition, copies of the pertinent sections of each of these references are attached hereto for the Examiner's consideration as Exhibits A and B, respectively. These references disclose experimental protocols regarding the manipulation of

these types of tissues. In contrast to the Examiner's contentions, these references, which are standard texts in the field, do not disclose the use of centrifugation at any point in the transformation process. This is because centrifugation is not necessary, and not because the description of this step is omitted.

Further, as amended, the centrifugal acceleration employed in the method of the present invention is 1000G to 150,000G. Even if Hansen centrifuged the type I callus or the immature embryos, Hansen does not employ a centrifugal acceleration as large as 1000G. It would be contrary to logic to infer that Hansen discloses the use of a centrifugal acceleration as large as 1000G to separate a plant tissue having a size of 1 mm or larger. That is, even when separating protoplasts having a size of several tens of micrometers, the centrifugal acceleration commonly used is only about 100G. (*See*, *The Maize Handbook*, Walbot, V.E. and Freeling, M., Eds, Springer-Verlag, N.Y., pp. 695 through 700, page 697, R.D. Shillito et al., "Maize Protoplast Culture" (1994); and Indra K.V. et al., "Embryogenic callus, cell suspension and protoplast cultures of cereals," *Plant Tissue Culture Manual*, Netherlands, Kluwer Academic Publishers, Supplement 1, B1/1-13 (1991), copies of the pertinent sections of each of which are attached hereto for the Examiner's consideration as Exhibits C and D, respectively). Thus, the conditions are not "identical" as the Examiner asserts on page 4 of the Office Action.

Any cited reference used for a rejection under 35 U.S.C. § 103(a) must be considered in is entirety, *i.e.*, as a whole, including those portions that would lead away from a claimed invention. (*See*, *W.L. Gore & Associates, Inc. v. Garlock, Inc.*, 220 U.S.P.Q. 303 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 851 (1984)). Therefore, the cited reference actually teaches away from the presently claimed invention because methods commonly used in the art did not use centrifugation at all. Even if one of ordinary skill in the art did use centrifugation, it would be at a low force of 100G. Applicants respectfully submit that it is not *prima facie* obvious to modify Hansen to include centrifugation at such a high force as presently claimed unless the reference suggests an advantage to be gained from the modification. (*See*, *In re Sernaker*, 217 USPQ 1, 6 (Fed. Cir. 1983)).

Thus, the presently claimed invention cannot be anticipated nor obvious in light of the disclosure of Hansen because Hansen does not disclose nor even suggest, all of the limitations as recited in amended claim 1. (*See*, *In re Vaeck*, 947 F.2d 488, 20 U.S.P.Q.2d 1438 (Fed. Cir. 1991), stating "the prior art reference (or references when combined) must teach or suggest all the claim limitations.").

Additionally, because a centrifugal acceleration of about 100G is sufficient for separating plant tissues and plant cells, it follows that there is no motivation for one of ordinary skill in the art to employ a centrifugal acceleration force greater than 100G. In the present invention, Applicants discovered the unexpected result that transformation efficiency is greatly improved when the centrifugation is carried out at a large centrifugal acceleration.

Thus, it would not have been obvious for one of ordinary skill in the art to select the specific centrifugal forces cited in the present claims because one of ordinary skill in the art, following typical protocols in the prior art, would have only used very small centrifugal forces of only about 100G. The use of centrifugal acceleration of from 1000G to 150,000G would never have been considered by one of ordinary skill in the art. Furthermore, there is no motivation or suggestion that can be cited anywhere in the prior art to utilize such force.

Since Hansen does not disclose each and every limitation of the presently claimed invention, at least as recited in amended claim 1, Hansen cannot anticipate the presently claimed invention. "A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." (*See*, *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 U.S.P.Q.2d 1051, 1053 (Fed. Cir. 1987)).

Dependent claims 3-7, 12, 14, 15, 17, 18, 20, 21, 23 and 24 are also not anticipated as, *inter alia*, they depend from a non-anticipated base claim, claim 1.

Furthermore, since Hansen does not disclose or suggest the method of the present invention, especially as recited in amended claim 1, and since Hansen, or any other prior art, does not provide any motivation to so modify typical protocols which call for very low centrifugal forces, or no centrifugation at all (since it is not necessary), the presently claimed invention is also not obvious in light of the disclosure of Hansen, even when considered in light of the knowledge of one of ordinary skill in the art and even taking into consideration routine "optimization" of such known protocols.

Since no specific reasoning is provided by the Examiner for the rejection of dependent claims 3-9, 11, 12, 14, 15, 17, 18, 20, 21, 23 and 24, the dependent claims are also not obvious for, *inter alia*, depending from a non-obvious base claim, claim 1. That is, the dependent claims all incorporate all of the limitations of claim 1, including the limitation regarding the centrifugal force used in the method of claim 1. Thus, the dependent claims are non-obvious in light of the disclosure of Hansen, even if combined with the knowledge of one of ordinary skill in the art, because these claims also recite the use of such centrifugal forces in the method of the present invention.

Reconsideration and withdrawal of the anticipation rejection and/or obviousness rejection of claims 1, 3-7, 12, 14, 15, 17, 18, 20, 21, 23 and 24 are respectfully requested.

ENTRY OF AMENDMENTS

The amendments to the claims should be entered by the Examiner because the amendments are supported by the as-filed specification and drawings and do not add any new matter to the application. Additionally, the amendments should be entered since they comply with requirements as to form, and place the application in condition for allowance. Further, the amendments do not raise new issues or require a further search since the amendments incorporate elements from dependent claims into independent claims and/or are supported by the as-filed specification. Finally, if the Examiner determines that the amendments do not place the

application in condition for allowance, entry is respectfully requested since they certainly remove issues for appeal.

CONCLUSION

In view of the above amendment, applicant believes the pending application is in condition for allowance.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact Thomas J. Siepmann, Ph.D., Reg. No. 57,374 at the telephone number of the undersigned below, to conduct an interview in an effort to expedite prosecution in connection with the present application.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37.C.F.R. §§1.16 or 1.14; particularly, extension of time fees.

Dated: November 30, 2006

Respectfully submitted,

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Maize (*Zea mays* L.)

Bronwyn R. Frame, Tina Paque, and Kan Wang

Summary

Agrobacterium tumefaciens-mediated transformation is an effective method for introducing genes into maize. In this chapter, we describe a detailed protocol for genetic transformation of the maize genotype Hi II. Our starting plant material is immature embryos cocultivated with an *Agrobacterium* strain carrying a standard binary vector. In addition to step-by-step laboratory transformation procedures, we include extensive details in growing donor plants and caring for transgenic plants in the greenhouse.

Key Words: *Zea mays*; maize; genetic transformation; *Agrobacterium*-mediated; *Agrobacterium tumefaciens*; standard binary vector.

1. Introduction

Transgenic maize is among one of the first biotechnology crops globally commercialized. A number of gene delivery systems such as the biolistic gun, electroporation, silicon carbide whiskers, and *Agrobacterium tumefaciens* infection can be used for maize transformation *(1)*. One of the greatest advantages of using the *Agrobacterium*-mediated transformation method is its ability to generate large numbers of maize events with single or relatively low transgene copy numbers *(2–4)*. In general, simple transgene insertion is preferred because these transgenic plants are less prone to multisequence-induced gene silencing *(5)* and have been shown to maintain higher and more stable transgene expression over generations *(4)*. However, one of the major challenges in implementing this method is that it involves balancing interactions between two living organisms, the plant and the bacterium, to achieve success. Cells of one maize genotype or tissue type may be transformable using the biolistic gun but not readily amenable to transformation using the *Agrobacterium* method if they are not susceptible to infection by this biological

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3. In a large (150 × 15-mm) sterile Petri plate, cut off the top 1 to 2 mm of kernel crowns using a new (sharp) scalpel blade (*see* **Note 25**).
4. Insert the end of a sharpened spatula straight down (do not insert at an angle or you may split the embryo in half) between the endosperm and pericarp at the basipetal side of the kernel (toward the bottom of the cob).
5. Pop the endosperm out of the seed coat by gently wiggling the spatula. (If you dislodge the kernel from the cob instead, you have inserted the spatula too deeply.) This exposes the untouched embryo, which is then gently coaxed onto the spatula tip and transferred directly to liquid infection medium.

3.4. Infection, Cocultivation, and Resting

1. Dissect up to 100 immature zygotic embryos directly into a 2-mL Eppendorf tube filled with bacteria-free infection medium (with 100 μ*M* AS).
2. Remove this first wash using a 1-mL pipetman, and wash embryos a second time with the same medium (1 mL).
3. After removing the final wash, add 1 mL of precultured *A. tumefaciens* suspension (adjusted to OD_{550} = 0.30 to 0.40 using a spectrophotometer) to the embryos. The tube is gently inverted (not vortexed) 20 times before resting it upright for 5 min on the bench with embryos submerged.
4. After infection, transfer embryos, along with the *A. tumefaciens* suspension to the surface of the cocultivation medium (300 mg/L L-cysteine) using a 1-mL pipet tip. (Cut off to enlarge the bore size.) Carefully pipet off any excess *A. tumefaciens* suspension surrounding the embryos using an uncut tip.
5. Orient infected embryos scutellum side up with the aid of a dissecting scope. Wrap plates with vent tape and incubate at 20°C (dark) for 3 d.
6. Transfer all embryos from the cocultivation medium to resting medium (35 embryos per plate). Wrap plates with vent tape and incubate at 28°C (dark) for 7 d (*see* **Note 26**).

3.5. Selection for Putative Transgenic Callus Events

1. After the 1-wk resting period, transfer all embryos to selection I medium (1.5 mg/L bialaphos) to begin selection. Plates are wrapped with parafilm and incubated at 28°C (dark).
2. Two weeks later, selection pressure is enhanced by transferring embryos to selection II medium (3 mg/L bialaphos) for two subcultures of 2 wk each.
3. About 6 wk after infection, a rapidly growing, embryogenic sector of callus emerges on a subset of infected embryos while no further callus proliferation, and in some cases browning, occurs on the majority of the other embryos. Each of these proliferating calli is considered an independent putative transgenic event (*see* **Note 27**).
4. Subculture each putative event to its own plate of selection medium II for maintenance and naming.
5. Molecular biological analyses such as Southern or Northern blot hybridization or histochemical β-glucoronidase (GUS) assays can be performed at this stage.

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Transformation of Maize Via *Agrobacterium tumefaciens* Using a Binary Co-Integrate Vector System

Zuo-yu Zhao and Jerry Ranch

Summary

This chapter describes a stepwise protocol to achieve success in genetic transformation of maize using *Agrobacterium tumefaciens* as a DNA delivery system. Researchers will be able to effectively transform immature embryos of Hi-II and related genotypes with this protocol. The outcome of the transformation process will be transgenic embryogenic callus tissue, transgenic plants, and transgenic progeny seeds. Recommendations for molecular confirmation and evaluation of transgenic tissue/plants are also provided.

Key Words: *Agrobacterium tumefaciens*; binary vector system; genetic transformation; maize; monocot; super-binary vector; transgenic corn; transgenic maize; transgenic plants.

1. Introduction

The rapid deployment of plant biotechnology has resulted in the development of genetic transformation systems for economically important crops. Genetic transformation of crops provides a powerful avenue for germoplasm improvement and production of superior commercial products as well as affording a tool for basic and applied research in plant sciences.

Agrobacterium, a natural plant pathogen, has been genetically modified and widely adopted as a DNA delivery system for genetic transformation in dicotyledonous and monocotyledonous plants. Maize, the third most planted crop in the world, can be transformed with *Agrobacterium (1–4)*. Highly efficient transformation of maize was achieved with *Agrobacterium* strains carrying a "Super-binary" vector *(1–3)*. Recently an *Agrobacterium* standard binary vector system was also successfully used to transform maize *(4)*. In general, *Agrobacterium*-mediated plant transformation offers the advantage that T-DNA

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3.2. Immature Embryo Preparation

1. Aseptically dissect embryos from caryopses and place in a 2 mL microtube containing 2 mL PHI-A following standard methods for maize.

3.3. Agrobacterium Infection and Co-Cultivation of Embryos

3.3.1. Infection Step

1. Remove PHI-A with 1 mL micropipettor and add 1 mL *Agrobacterium* suspension. Gently invert tube to mix.
2. Incubate 5 min at room temperature.

3.3.2. Co-Culture Step (see Note 5)

1. Remove *Agrobacterium* suspension from infection step with 1 mL micropipettor.
2. Scrape the embryos from the tube using a sterile spatula.
3. Transfer immature embryos to plate of PHI-B medium in a 100 × 15 mm Petri dish. Orient the embryos with embryonic axis down on the surface of the medium.
4. Culture plates with embryos at 20°C, in darkness, for 3 d.
5. Transfer embryos to PHI-C with the same orientation and incubate at 28°C for 3 d.

3.4. Selection of Putative Transgenic Events

1. Transfer 10 embryos to each plate of PHI-D selection medium in a 100 × 15-mm Petri dish, maintaining orientation. Seal dishes with Parafilm.
2. Incubate plates in darkness at 28°C. Actively growing putative events, as pale yellow embryogenic tissue, should be visible in 6–8 wk. Embryos that produce no events may be brown and necrotic, and little friable tissue growth is evident.
3. Subculture putative transgenic embryogenic tissue to fresh plates of PHI-D at 2–3 wk intervals, depending on growth rate. Record events.

3.5. Regeneration of T_0 Plants

1. From embryogenic tissue propagated on PHI-D, subculture tissue to somatic embryo maturation medium, PHI-E, in 100 × 25 mm Petri dishes.
2. Incubate plates at 28°C, in darkness, until somatic embryos mature, for about 10–18 d.
3. Individual, matured somatic embryos with well-defined scutellum and coleoptile are transferred to PHI-F embryo germination medium and incubated at 28°C in the light (about 80 µE from cool white or equivalent fluorescent lamps).
4. After shoots and roots emerge, about 7–10 d, individual plants are transferred to PHI-F medium in 150 × 25 mm glass tubes covered with closures, and incubated at 28°C in the light (about 80 µE from cool white or equivalent fluorescent lamps).
5. In 7–10 d, regenerated plants, about 10 cm tall, are potted in horticultural mix and hardened-off using standard horticultural methods.

Michael Freeling Virginia Walbot
Editors

The Maize Handbook

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careful attention is given to developing the callus cultures and selecting strongly for the best suspension culture morphology. Regenerate a sample of the established suspension to check that it has retained its ability to regenerate fertile plants. The number of plants regenerated and the fertility of the plants are likely to be lower than those seen for the callus from which the suspension was derived.

Monitor the suspension every 2–3 weeks for release of protoplasts. When a yield of 5–10 million per g fresh weight is obtained, begin plating the protoplasts to monitor division and colony formation.

Cryopreservation can be used to maintain a supply of regenerable embryogenic suspension cultures with known behavior and to eliminate the need to continually initiate fresh culutres. Suspension cultures can be cryopreserved easily and will form new suspensions suitable as a source of protoplasts within 1–3 months of recovery from the freezer. Take samples of the established suspension culture every 4–6 weeks for cryopreservation (DiMaio and Shillito 1989). In this way, you will have a sample of the culture at the optimum stage in its development when it is subsequently needed to initiate new cultures. Callus can also be cryopreserved, with varying success, depending on its morphology. Good friable cultures cryopreserve well, whereas type II cultures are difficult to cryopreserve.

ISOLATION OF PROTOPLASTS

Incubate 1–1.5 ml PCV of the suspension culture cells in 10–15 ml of a mixture consisting of 4% w/v RS cellulase with 1% w/v Rhozyme in KMC (8.65 g/liter KCl, 16.47 g/liter $MgCl_2$. $6H_2O$ and 12.5 g/liter $CaCl_2$. $2H_2O$, 5 g/liter MES pH 5.6) salt solution. Two percent w/v macerase can also be added if desired and may increase the number of protoplasts released. Carry out digestion on a rocking table for a period of 3–4 hours. Monitor the release process under an inverted microscope. Young cultures will generally take longer to release protoplasts.

Collect the released protoplasts as follows: Filter the preparation through a 100-μm mesh sieve followed by a 50-μm mesh sieve. Wash the protoplasts through the sieves with a volume of KMC salt solution equal to the original volume of enzyme solution. Place 10 ml of the protoplast preparation in each of several plastic disposable centrifuge tubes and layer 1.5–2 ml of 0.6 M sucrose solution [buffered to pH 5.6 with 0.1% w/v morpholino-ethane sulfonic acid (MES) and KOH] underneath. Centrifuge at 60–100*g* for 10 minutes; collect the protoplasts banding at the interface and place them in a fresh tube.

Resuspend the protoplast preparation in 10 ml of fresh 13/14-strength KMC salt solution and centrifuge for 5 minutes at 60–100*g*. Remove the supernatant and resuspend the protoplasts gently in the drop of medium remaining, and then gradually add 10 ml of a 6/7-strength KMC solution. Remove an aliquot for counting and sediment the remaining protoplasts again by centrifugation. Resuspend the protoplasts at 2×10^7 per ml in KM culture medium (modified Kao and Michayluk 6p medium, containing double the

PLANT TISSUE CULTURE MANUAL

Supplement 1

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Culture of embryogenic protoplasts [19, 22, 23, 41–43]

Steps in the procedure

1. Best results are obtained when cells from a rapidly growing, finely dispersed embryogenic suspension culture are used during the exponential phase of growth. Approximately 1.5 ml of the settled cells are harvested 2–7 days after subculture and placed in 15 ml of a filter-sterilized enzyme solution in a Petri dish. The amount and combinations of enzymes used vary from species to species, and from cell line to cell line. However, the following may be used as a good starting point: 1–3% cellulase Onozuka RS, 1% pectinase Serva, dissolved in a buffer solution consisting of 7 mM $CaCl_2 \cdot 2H_2O$, 0.7 mM $NaH_2PO_4 \cdot H_2O$, 0.5 M mannitol, and 3 mM MES at pH 5.6 (osmolality adjusted to 650 m osm/kg H_2O). The cells are incubated at room temperature on a gyratory shaker at 50 rpm. Protoplast yields of 3–5 × 10^6/ml can be obtained within 4–6 hr. In some species, use of small amounts of Driselase and Pectolyase Y23 is necessary to obtain good protoplast yields. Incubation in the enzyme mixture for longer than 4–6 hours should be avoided. Commercially available enzymes can be used directly, without any additional purification.
2. The protoplast-enzyme mixture is filtered through a layer of Miracloth, and then successively through 100 µm and 25 µm stainless steel filters. The protoplasts are collected by centrifugation at 100 × g for 3–4 min, and washed three times with the wash solution (the enzyme solution without the enzymes, but containing 0.6–0.8 M mannitol).
3. Protoplasts are cultured in shallow layers (1.5–3.0 ml medium) in 6 cm Petri dishes at a density of 1–3 × 10^5/ml, either in liquid or 0.3–1.2% low-melting-point Seaplaque agarose (FMC) solidified Kao and Michayluk's [44] medium as modified by Vasil and Vasil [41], containing various concentrations of 2,4-D (there are some recent reports of good plating efficiencies obtained even in MS medium). In some species addition of cytokinins, like zeatin (0.1–0.2 mg/l), has been found to be beneficial. The cultures are incubated in the dark at 28 °C. Fresh nutrient medium with reduced osmoticum (0.3 M glucose) is added after 10–14 days. Protocolonies become visible within four weeks and can be transferred to fresh medium with 3% sucrose or 2% each of glucose and sucrose The use of feeder layers or floating sectors of agarose-plated protoplasts in liquid media (agarose bead culture) have been found to be particularly useful in maize [19, 43] and other species where plating efficiencies may be rather low, and in the selection of transformed cells.
4. Protoplast-derived embryogenic callus is transferred to regeneration media as described earlier to induce the formation of somatic embryos and plants, in much the same manner as that obtained from the culture of immature embryos, inflorescences, leaves and suspension cultures.